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THE FUNCTION AND STRUCTURE OF PERIPHERAL NERVES
FOLLOWING CUTANEOUS BURNS (U) WASHINGTON UNIV SEATTLE
SCHOOL OF MEDICINE W M MONAFO 15 JUN 83

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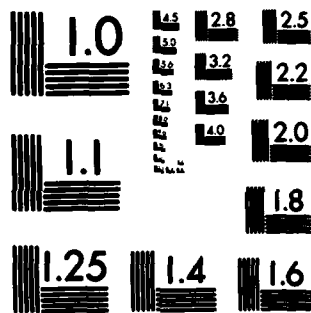
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MICROCOPY RESOLUTION TEST CHART
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Contract No. DAMD17-82-C-2097
William W. Monafo, M.D., Principal Investigator
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THE FUNCTION AND STRUCTURE OF PERIPHERAL NERVES
FOLLOWING CUTANEOUS BURNS

Annual Summary Report

15 June 1983

William W. Monafo, M.D.

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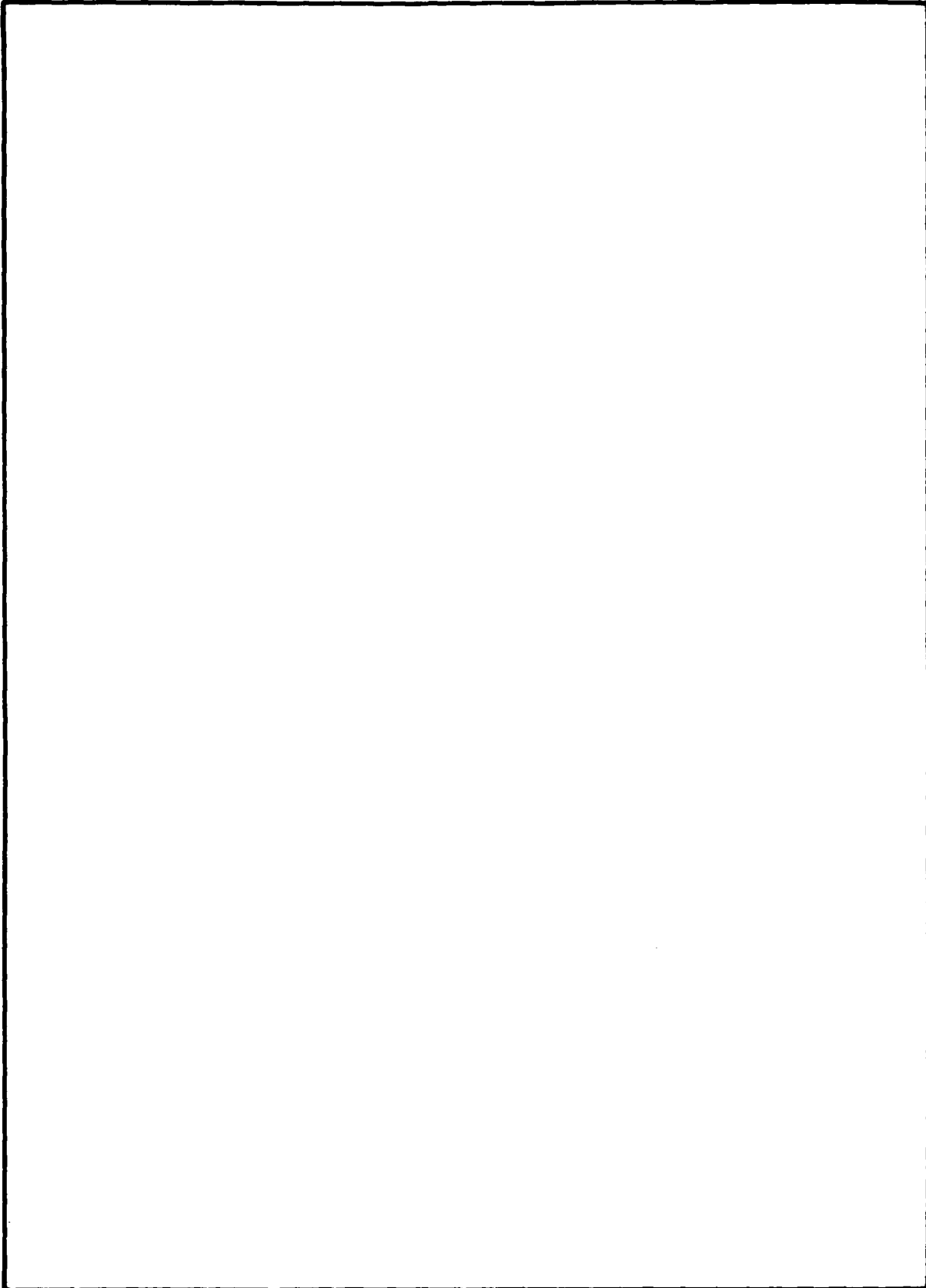
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SUMMARY

Using a radio frequency generator several experimental models have been developed which permit study of the effects of heat on the electrophysiological function, the biochemistry and the morphology of the rat sciatic nerve and its major branches. Experiments are carried out both in vivo and in vitro. The results to date indicate that motor fibers are more resistant to a given heat load in vitro than are sensory ones of similar diameter. With respect to fiber size, the A-delta groups appear to be more sensitive than are the A-alpha fibers. C-fiber heat sensitivity is apparently intermediate between the A-delta and A-alpha. Biochemical analyses of injured nerves indicate striking changes both in the myelin and soluble protein fractions. The time course of neural edema formation and resolution and of endoneurial vascular permeability change is being correlated with the biochemical, structural and electrophysiological abnormalities observed.

FOREWORD

Little is known about the etiology and pathogenesis of the peripheral neuropathies which become evident clinically in a significant proportion (20-30%) of patients with major thermal injuries. This project studies the effects, direct or indirect, of thermal injury on the electrophysiological function and associated biochemical and morphological alterations of the sciatic nerve that attend thermal injury in rats.

APPENDIXES

TABLES

Table 1: Chamber 45° X 4 Min, RF 1 MHz, Mean Body Weight = 264.6 g (N=6), Time

Table 2A: Chamber 47° X 4 Min, RF 1 MHz, Mean Body Weight = 267.1 g (N=11), Sham-control (Contralateral to burn)

Table 2B: Chamber 47° X 4 Min, RF 1 MHz, Mean Body Weight = 267.1 g (N=11) Burned Side

FIGURES

Figure 1: See text for description.

Figure 2: See text for description.

Figures 3a,3b,3c: Acrylamide gel electrophoresis. The three gels from nerve to the left are soluble, myelin and particulate protein fractions. The three gels to the right are from sciatic nerve burned in vitro at 45°C for 14 minutes and then immediately analyzed. Abnormal bands are present on the right, but are difficult to distinguish here. The same preparation was subjected to HPLC, the results of which are given in Figure 3b and 3c for soluble protein and myelin basic protein.

Several approaches have been used to develop appropriate models for the assessment of the effects of heat on peripheral nerve function. Initially, water immersion scalds were employed. Implanted thermistors, however, showed that the temperature reached in the vicinity of the sciatic nerve in the popliteal space, where the injury was centered, was highly variable. It was found that circumferential immersion of the hind limb in water at 90°C for 20 seconds with the paw shielded was required in order to produce a clinically evident foot drop by the following day in 50 to 60% of the animals. However, this consistently resulted in full thickness circumferential skin loss and obvious necrosis of a varying bulk of the underlying muscle. In addition, paw swelling was sometimes intense, which interfered significantly with percutaneous measurements of nerve conduction velocity, which was found normally to average 36.9 meters/second \pm 3.4 (S.D.). Terminal latencies in control animals show a narrow and acceptable range of variability (1.15 millisecond \pm 0.18 (S.D.)). It was found that the sciatic nerve in animals who apparently had a conduction block, as indicated by percutaneous measurement, actually functioned in vitro when the nerve was immediately excised and placed in an Harvard chamber, where it could be directly stimulated and sensory as well as motor nerve excitability assessed. In addition, this approach was unsatisfactory because of the obvious infection that developed in the large open wounds in some instances, which required sacrifice of the animals for humane reasons. The range in temperatures reached at the sciatic nerve was from 38° to 45°C, baseline being approximately 32°C. These findings were attributed to variability in limb blood flow, which could not be controlled. Subsequently, therefore, a series of 10 experiments was performed using a thermostatically heated brass block, which was positioned on the skin overlying the popliteal space. Thermistors were inserted percutaneously through plastic cannuli into the region of the nerve. The heat was applied after percutaneous control measurements of motor nerve conduction velocity had been obtained. As was the case with the circumferential immersion scalds, a severe injury of the underlying skin and muscle resulted when the heat was applied for a period long enough to produce an immediate change in the amplitude of the continuously displayed action potential of at least 50%. Also again, presumably due to inherent variability in blood flow, the rate of rise of temperature in the region of the nerve varied unacceptably and in an almost random manner.

For these reasons, it was judged necessary to utilize a heat source whose output could be readily varied. After consultation with Dr. Gilbert Nussbaum of the Section of Radiation Physics, it was elected to utilize a radiofrequency generator to permit controlled ionization of the applied heat load and appropriate similar time-temperature profiles in experimental groups. This approach has been utilized and refined since October 1, 1982. Several chambers have been designed, most of them using horizontally aligned copper electrodes of varying sizes. A small chamber, designed for direct implantation in in vivo experiments on the exposed sciatic nerve or its major branches, and which can be utilized as well in in vitro studies with the nerves placed in an Harvard chamber in various configurations, was constructed and has been used extensively subsequently. Larger chambers, which permit the percutaneous application of the RF current have also been built in sizes appropriate to contain the intact hind limb in the region of the popliteal space. By the simple expedient of irrigating the skin within the confines of the chamber with room temperature or iced isotonic saline, cutaneous injury can be nearly always eliminated at the same time that the temperature in the region of the main trunk of the sciatic nerve, as measured by a percutaneously implanted thermistor, can be

elevated to the desired extent; moreover, the rise time of the temperature in both the large and small chambers can be regulated within an acceptable range.

A series of experiments was then carried out on intact, nembutal-anesthetized animals whose sciatic nerve was exposed and freed from the surrounding adventitia in the proximal portion of the popliteal space just proximal to the takeoff of the peroneal nerve, where the small chamber was positioned over the nerve and a thermistor probe was placed in contact with the epineurium. After control measurements of nerve function had been obtained, the nerve temperature was raised to 45° within 60 - 80 seconds and this temperature maintained for 4 minutes. The results are shown in Table 1 (Appendix). Measurements were taken immediately after the tissue temperature had returned to 32 - 34°C following the heat load, and at 1, 2, and 6 days subsequently. As can be seen, there was no apparent change in motor function. Clinically, none of the animals evidenced a foot drop. Histologic examination of the nerves at the level of injury (and proximally and distally as well) was normal. Nerve water content remained at the control level of 69.5%. Contralateral, sham-operated nerve, into which the chamber was implanted, but RF current not applied, showed no perceptible changes (Table 1).

In another, similar series of experiments, chamber temperature was elevated to 47°C and also maintained for 4 minutes. Nerve function was remeasured immediately following injury and subsequently at 1, 2, 9, and 14 days later.

RESULTS (TABLES 2A and 2B): These results contrasted markedly with those that obtained at 45°C. A complete motor nerve conduction block was present in 3 of 11 animals immediately after the thermal load had been applied. In the remainder, a marked fall in the amplitude of the action potential, as recorded distal to the injury site, was immediately apparent in 5 of the remaining animals. By 24 hours, a reduction in amplitude with both proximal and distal stimulation was evident in all rats. These changes persisted for as long as 14 days (Table 2B). Conduction velocities in those animals in whom an action potential could still be evoked by maximal stimulation also appeared to be prolonged during the follow up period.

Histologic examination at 24 hours of the injured area of nerve disclosed separation of individual myelinated fibers within the nerve itself, consistent with edema. In the more severely affected areas, the normal anatomic features of the endoneurium and unmyelinated fibers was obscured by amorphous staining material, probably representing protein-containing edema fluid. There was marked congestion of endoneurial and perineurial vasculature. Individual myelinated fibers were extensively and unselectively damaged. The axons were swollen and clear, with the myelin sheaths being in varying stages of disintegration. The axons in general appeared to be more severely affected than the myelin sheaths. Examination at 14 days post injury disclosed evidence of regeneration with the formation of bands of Schwann's cells. There were a few intact myelinated fibers present. The regenerative activity appeared widespread and morphologically proceeding along the usual lines.

The neural edema was present at both 24 hours and 14 days post injury. Nerve water content was 74.07% and 77.36% on Day 1 and 14 respectively in the burned nerve. The corresponding values in the contralateral uninjured nerve were 72.44% and 70.88%.



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As noted above, in early experiments we established standard values for percutaneously measured conduction velocity and evoked potential amplitude. We have noted, however, that these determinations in scald burns and percutaneous burns can be very deceptive. The amplitude variations are easily affected by the presence of edema and bear no relation to the actual decrease in number of active nerve fibers. Conduction velocity measurements may also be difficult, either because the edema around the recording foot electrode diminishes the response or distorts it, or -- occasionally -- because current is short-circuited to the recording point and conduction velocities of improbable magnitude are measured. This is of some practical importance because the cause of weakness in a burn patient may be misinterpreted if based on routine electromyographic examination. In order to pursue the question of differential sensitivity (sensory vs motor fibers), a series of in vitro electrophysiological preparations were examined.

Multiple sets of experiments have been carried out in vitro (Harvard Chamber) to evaluate the effect of heat on the ability of the sciatic nerve complex to carry impulses. In the first sequence, stimulation was applied to the sciatic nerve and recordings taken from the peroneal nerve, which has predominantly motor fibers, and from the sural nerve, which is predominantly a sensory nerve. Although these two nerves have different functions, their composition in regard to fiber size spectrum is quite close. The nerves were evaluated in vivo prior to removal, and their terminal latencies and conduction velocities were within the limits previously established. The nerves were then removed to a Harvard chamber and a small, slotted, RF heating chamber utilizing copper electrodes was applied around the nerve. Starting temperature ranged from 32°C to 34°C; chamber temperature was raised to 45°C, temperature rise time varying between 26 and 138 seconds. Due to interference, it was usually necessary to interrupt the heating for a few seconds in order to record the evoked potentials in the two nerve branches. This was repeated every two minutes until the amplitudes in the longer-lasting nerve was reduced to near-zero (less than 10% of its original value). The amplitude decay was plotted against the time of the heating. Linear regression analysis was applied to the amplitude decay curves. The decay curves are listed below:

<u>Peroneal</u>	<u>Sural</u>
$Y = 0.71 - 0.62X$	$Y = .14 - 0.03X^*$
$Y = 5.3 - 0.79X$	$Y = .26 - 0.02X$
$Y = 4.0 - 0.28X$	$Y = .64 - 0.07X$
$Y = 6.2 - 0.47X$	$Y = 0.8 - 0.04X$
$Y = 2.63 - 0.17X$	$Y = 0.69 - 0.04X$
$Y = 6.6 - 0.81X$	$Y = .64 - 0.06X$

Where Y = amplitude (mV), X = Time (Min)
 (R ≥ 0.95 in all but the starred instance)
 * r = 0.64

Evidently, the values for the sural nerves are quite different from those in the peroneal nerve. Judging from the Y intercept, some nerves were in better condition than others, possibly due to differences in preparation and cleaning of the nerve. The distinct difference in these slopes is indicated by the 10 to 30 fold less negative coefficients in the sural nerves as compared to the peroneals.

Certain characteristics of these amplitude decay curves become apparent upon closer analysis. The following features are of special interest:

1. In several cases a period of facilitation, i.e., increased amplitude was noted in the early stage of heating.
2. Several of the preparations showed prolonged resistance to the heat, with the amplitude remaining constant over several minutes. In order to explore in greater detail the changes that occurred in the two nerves, the experimental design was altered as follows: as it is clear that the heating of the common sciatic trunk involves the separate heating of the fascicles, including those for the peroneal and the sural as well as the posterior tibial, uneven application of heat is likely. Second it is difficult to judge the uniformity of the heat on the surface of the nerve vs the center of the nerve. It is conceivable that the initial application of heat creates an area of depressed neural activity which surrounds the remaining fibers as an isolating ring leaving the central nerve fibers intact to carry on until heat penetrates further. We therefore rearranged the heating chamber so that cleaned and stripped nerves would be heated and recording would also take place from the stripped area of the sural and peroneals respectively. With this preparation, the decay of the amplitude of the action potential in the peroneal nerve decayed at a somewhat lesser rate, with an average survival time of 20 to 22 minutes, whereas the sural nerve action potential had a somewhat complex form and decayed over an average of 12 to 14 minutes (Fig. 1).

The form of the decay curves is particularly interesting. The facilitation is seen more frequently in the sural nerve but occasionally also in the peroneal nerve. Because in each experiment attempts are made to use an activation intensity of at least twice the maximal needed, and because the increase is not accompanied by a shift in the shape of the action potential, the most likely explanation is repetitive firing. The possibility of such a direct heat effect was hinted at in earlier work by Zimmerman (1). This phenomenon clearly requires study, however. The previously discussed plateau formation, with the action potential remaining constant over several minutes at 45°C, appears to be more pronounced when the nerve is less well prepared or cleaned. It seems likely that the heat applied could be disseminated among residual connective tissue. As can be expected from the indicated average survival times, the slope of the regression curves from the sural nerve group is more steep than that for the peroneal nerves (Fig. 2). We plan to continue studying the differences between the motor and sensory nerves in preparations of the motor and sensory tail nerves and motor and sensory roots. Should the difference between the two types of nerves turn out to be a generalized phenomenon, it would be appropriate to follow up with an analysis of the polynomial curves ($Y = Z^x (1-Z)^{n-x}$) that can be fitted to the exponential decay. The mathematical background for such analysis is available in the literature. One might, at this time, hypothesize that the applied heat changes the longitudinal distribution of the electrotonic potentials and/or the membrane current. It is impossible at this stage to exclude ionic shifts possibly caused by effect of the heat on the protein matrix and subsequent change in pore size in the nodal and axonal membranes.

We have studied the effect of varying the rate with which the temperature was raised from 34°C to 47°C at which level it was maintained for 30 seconds. The behavior of the peroneal nerve compound action potential was erratic. The amplitude of the sural nerve action potential was inversely proportional to the rate of temperature increase. These experiments require refinement using a smaller

temperature chamber and isolated fiber fascicles from the sural nerve and peroneal nerve respectively. It will also be necessary to overcome a technical problem of overshoot with rapid rise times.

We have initiated studies on the differential sensitivity of fibers of different size from the same nerve. Using a monopolar recording technique with the one end of the nerve killed by compression and application of .11 molar potassium chloride action potentials are obtained that permit analysis of the effect of heat on large, medium size and smaller nerve fibers. Preliminary observations indicate that A delta group is the most sensitive, and that these fibers first slow down their conduction velocity and then cease to conduct. A alpha fibers are, in our experimental situation, more persistent. This would be in line with the observations by Goldring and Fletcher of the relatively better resistance of larger fibers in in vitro experiments (2). We believe that our approaches will permit us eventually to explain the contrasting results by Zimmerman's group in which A fibers cease to function before C fibers. For instance, the complex arrangement utilized by Zimmerman with multiple heating and cooling in vivo with an intact circulation presents a different picture in which the above-referred to isolating effects of non-neural tissue may play an important part.

Good progress has been made in the study of chemical changes that take place in the segment of the nerve that is being heated. Our first experiments used in vitro immersion of nerves comparing controls with immersion in four minutes at 45°C and 48°C. The immersed portion of the nerve was analyzed after homogenization and delipidization by extraction of the protein and subsequent separation on vertical acrylamide slabs. A number of new bands are present in the lower molecular weight region of the gel. Our more recent experiments have also made use of the radio frequency generator in order to parallel our physiological studies. The heated segment is removed and frozen and the proteins separated in myelin, soluble, and particulate protein fractions (Fig. 3). A portion of each sample has then been filtered by centrifugation through a nylon filter and analyzed with high pressure liquid chromatography on Spherogel gel with phosphate buffer containing SDS. Representative curves are attached indicating a striking shift both in the myelin fraction and the soluble protein fraction (Fig. 3b and 3c). Myelin basic protein is broken down to smaller fragments and it is probable that these are the ones that are appearing in the soluble protein fraction. These poly- and oligopeptides may well have a dual affect. First, it is possible that they themselves may act immediately to induce conduction block and conduction velocity decrease. Secondly, it is possible that they, over a period of days, may be responsible for more serious structural effects. The possibility of antibody formation, for example, must be considered. Heat may impair axonal flow, an event also probably detrimental to the recovery of the nerve.

We have currently going a series of long-term experiments with application of heat and followup of the action potentials in vivo and in vitro. The clinical state, sensitization by previous drugs and the effect on the contralateral extremity are being investigated. In these experiments we also compare the application of heat to isolated nerve segments to the application of heat percutaneously to a larger muscle bulk surrounding the nerve, which more closely resembles the clinical situation. The use of radio frequency heating with simultaneous cooling of the skin permits long-term studies without serious interference from skin damage, breakdown, infection, and mutilation.

REFERENCES

1. Klumpp D, Zimmermann M: Irreversible differential block of A- and C-fibers following local nerve heating in the cat. J Physiol 298:471-482, 1980.
2. Letcher FA, Goldring S: The effect of radio frequency current and heat on peripheral nerve action potential in the cat. J Neurosurg 29:42-47, 1968.

TABLE 1

CHAMBER 45° X 4 MIN, RF 1 MHz

Mean Body Weight = 264.6 g (N=6)

	<u>SHAM</u>		<u>TIME</u> <u>BURN</u>			
	<u>Pre-injury</u>	<u>24 hrs.</u>	<u>Pre-injury</u>	<u>24 hrs</u>	<u>48 hrs</u>	<u>6 days</u>
Conduction Velocity M/sec	36.00	36.02	40.58	33.38	34.15	38.05
Proximal Amplitude* mm	33.25	25.98	31.03	25.92	21.30	29.10
Distal Amplitude* mm	40.01	33.02	34.15	30.36	28.10	36.00

*1 mV = 5.52 mm

TABLE 2A

CHAMBER 47° X 4 MIN, RF 1 MHz

Mean Body Weight = 267.1 g (N=11)

SHAM-CONTROL (CONTRALATERAL TO BURN)

	<u>Pre-injury</u>	<u>24 hrs.</u>	<u>48 hrs.</u>	<u>9 days</u>	<u>14 days</u>
Conduction Velocity M/sec	42.8	32.5	37.8	73.6	22.13
Proximal Amplitude* mm	38.2	32.8	25.0	26.7	26.6
Distal Amplitude* mm	44.5	43.6	29.8	33.8	35.6

*1 mV = 5.52 mm

TABLE 2B

CHAMBER 47° X 4 MIN, RF 1 MHz

Mean Body Weight = 267.1 g (N=11)

	<u>BURNED SIDE</u>				
	<u>Pre-Injury</u>	<u>24 hrs</u>	<u>48 hrs</u>	<u>9 days</u>	<u>14 days</u>
Conduction Velocity M/sec	37.8	17.2	10.0	20.7	26.5
Proximal Amplitude* mm	26.9	3.08	0.74	0.9	1.1
Distal Amplitude* mm	30.6	5.9	0.6	0.8	3.2

*1 mV = 5.52 mm

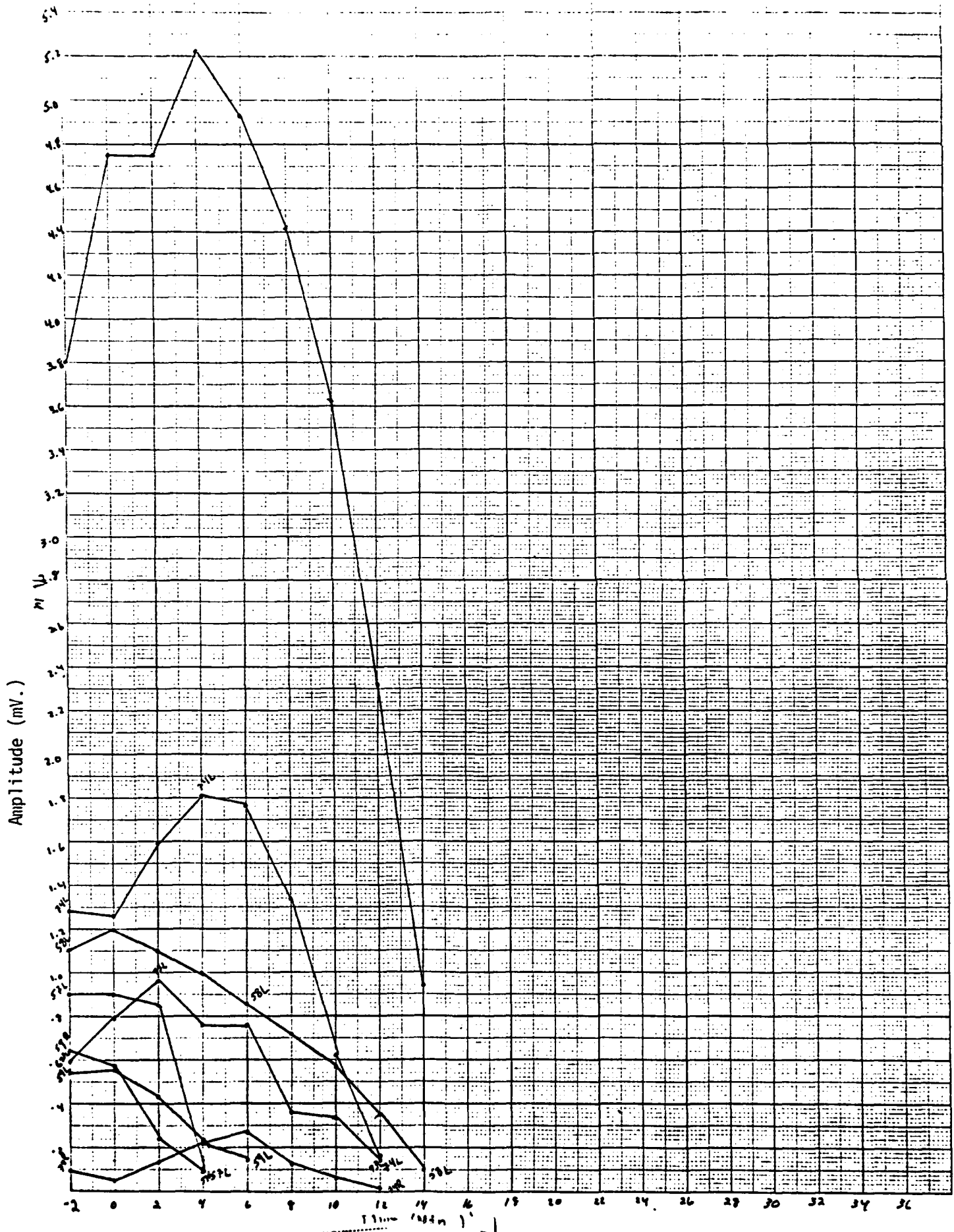
W. L. Kelley, Jr., Editor, *Mathematics*



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Principal Investigator
Fig. 1

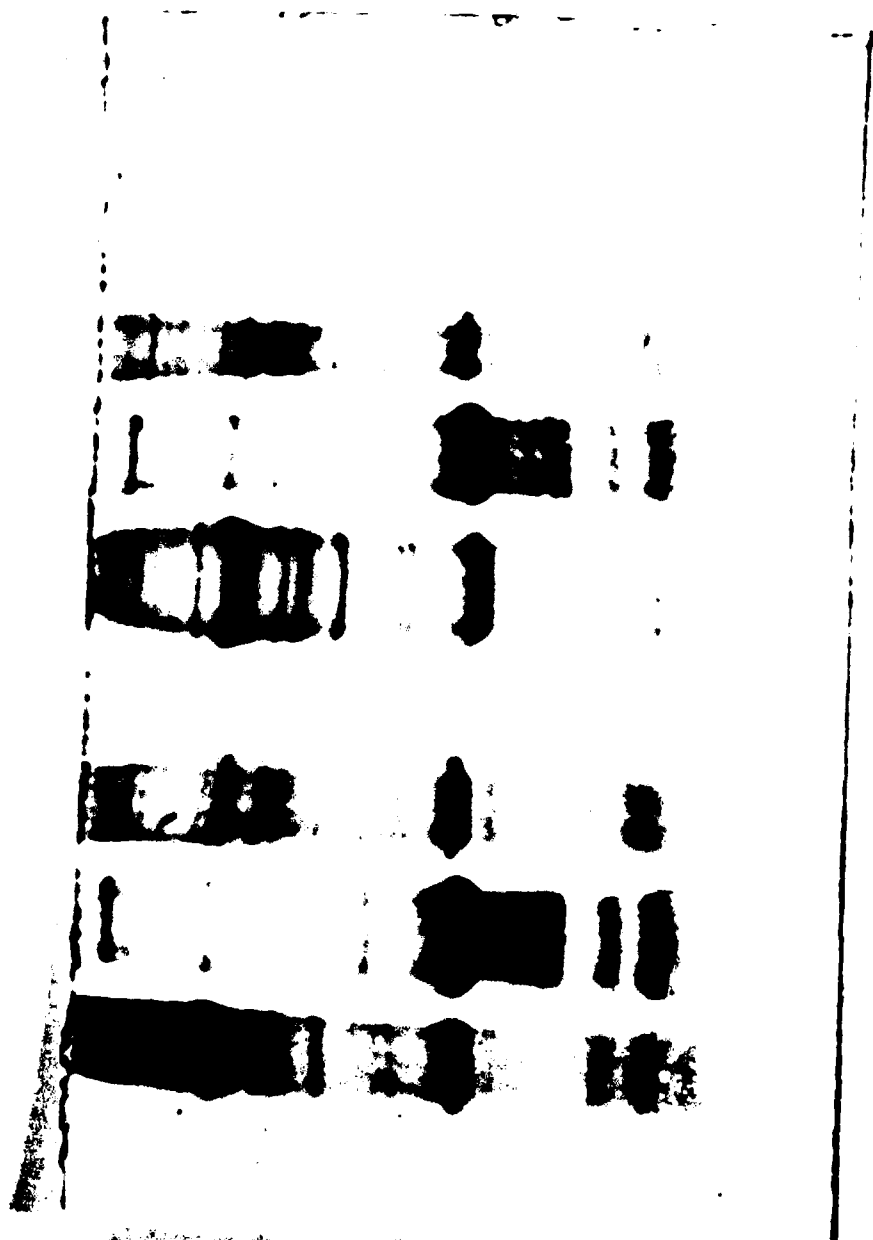
Clean Surals in Chamber (burn at 45°C)



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Principal Investigator
Figure 2



70P
A

Fig 3a

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Principal Investigator
Figure 3a

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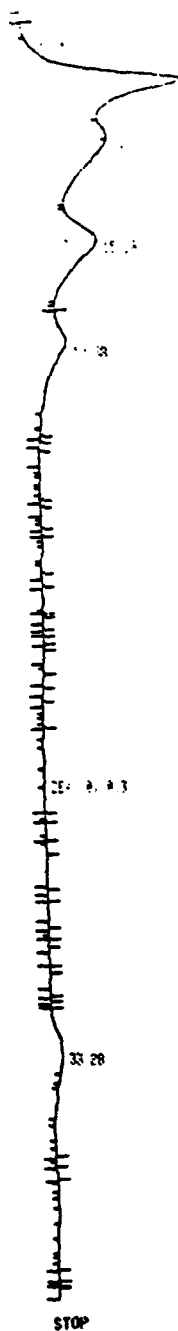
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A. Control rat
Soluble protein
65 ug
280 mu

B. 45°C burn

Soluble protein
46,8 ug
280 mu

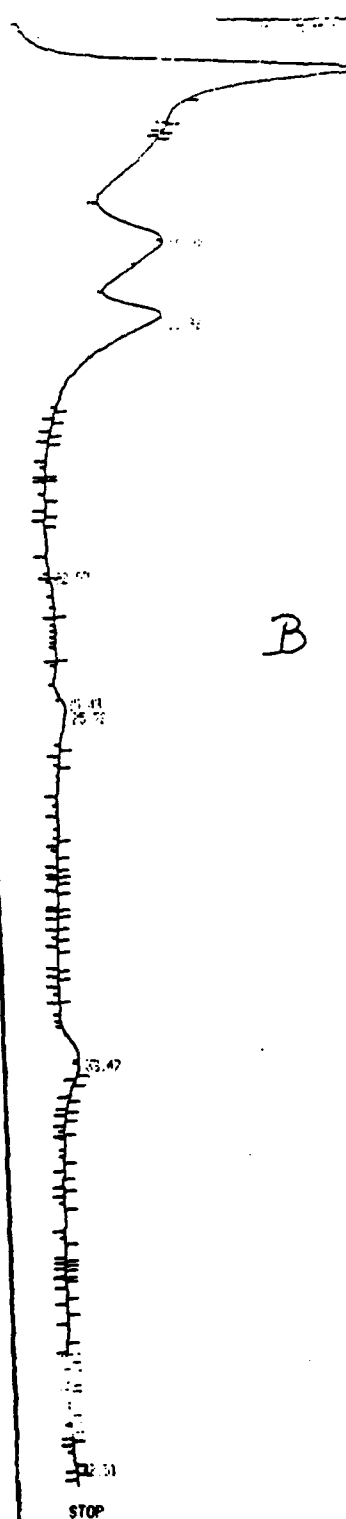
Note:
Shift of
proteins
from peak
at 11.5 min.



A

AREA%	RT	AREA TYPE	AR/MT	AREA%
10.60	10090	BV	0.115	0.001
11.54	5911300	VV	0.813	53.001
12.78	1404700	VV	0.408	12.594
15.19	2651300	VV	1.131	24.175
17.38	653240	BP	0.825	5.857
33.28	477700	BV	0.942	4.283

TOTAL AREA= 1.1157E+07
NUL FACTOR= 1.0000E+00



B

RUN 0 37

MAY/12/83 10:53:36

AREA%	RT	AREA TYPE	AR/MT	AREA%
2.09	13640	PP	0.021	0.036
10.59	340	PP	0.017	0.009
11.46	853200	PV	0.142	22.232
15.22	640080	BV	0.370	16.671
16.92	2067000	VV	1.002	54.225
22.57	5309	PP	0.213	0.138
25.43	18617	PV	0.140	0.405
25.72	135900	VV	0.603	3.540
33.47	100000	PV	0.494	2.607
42.31	2217	PS	0.192	0.058

TOTAL AREA= 2837500
NUL FACTOR= 1.0000E+00

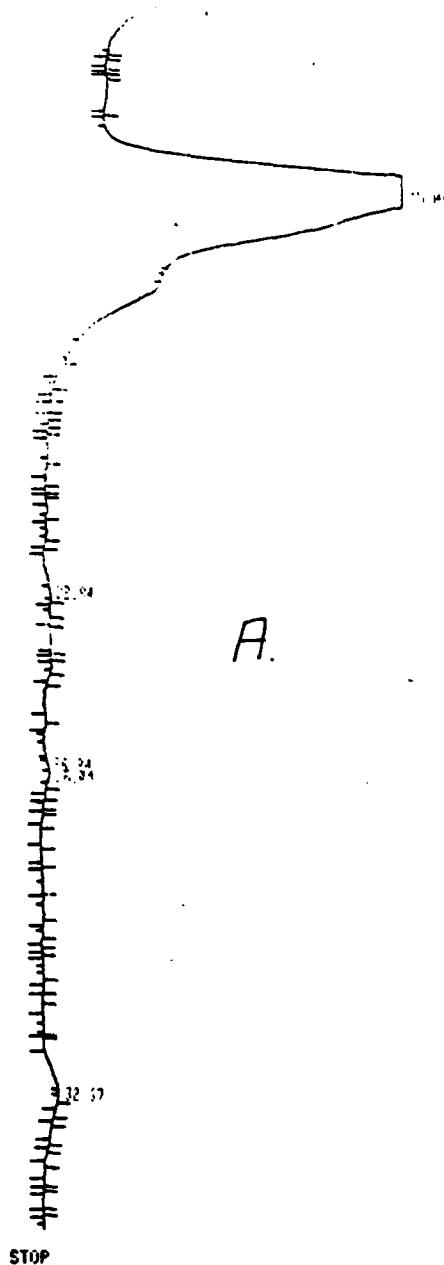
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CONTRACT DAMD17-82-C-2097
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Figure 3b

A. Control rat
Myelin fraction
29 ug
280 mu

B. 45°C burn
Myelin fraction
53 ug
280 mu

Note: Basic
protein has
broken into
4 separate
low mol. weight
peaks.

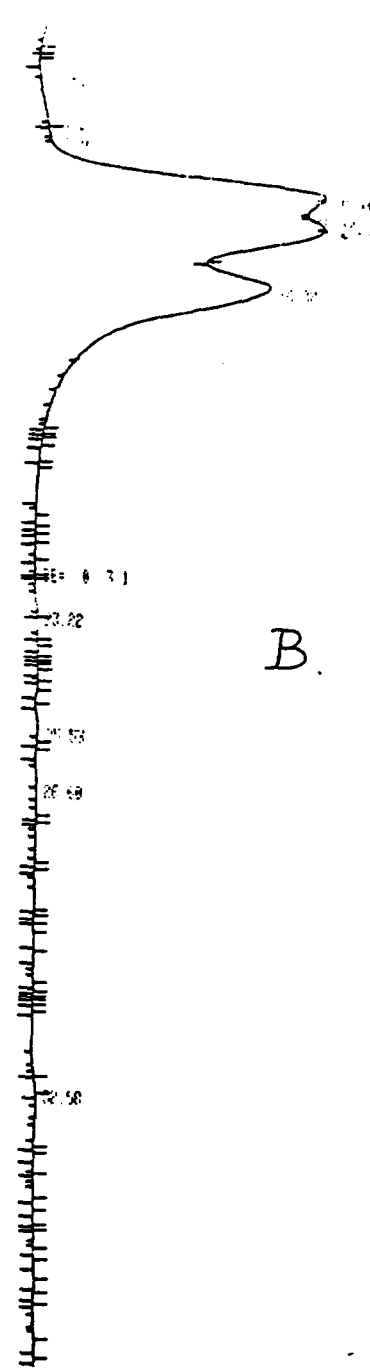


STOP

RUN # 28 MAY/09/83 12:35:07

RT	AREA	TYPE	AM/HT	AREA%
11.50	1282000	PV	0.696	10.362
15.81	1.1844E+07	VV	1.243	89.197

TOTAL AREA= 1.2177E+07
MUL FACTOR= 1.0000E+00



STOP

RUN # 27 MAY/09/83 11:54:40

RT	AREA	TYPE	AM/HT	AREA%
13.61	18278	PP	0.395	0.654
13.92	38504	PP	0.150	0.835
15.04	3480300	PV	0.498	31.006
15.42	3700000	VV	0.049	2.940
15.67	1601600	VV	0.238	14.269
15.70	2382200	VB	0.355	21.823
16.82	2813300	BP	0.715	25.063
23.22	6547	VP	0.280	0.058
25.53	74290	SV	0.285	0.216
26.60	9102	PV	0.289	0.081
32.58	4351	PB	0.207	0.039

TOTAL AREA= 1.1225E+07
MUL FACTOR= 1.0000E+00

TOP.



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Figure 3c

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